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TITLE: Dissecting the Roles of Brain Injury and Combat-Related Stress in Post-Traumatic Headache

PRINCIPAL INVESTIGATOR: KC Brennan

RECIPIENT: University of Utah
               Salt Lake City 84108

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                   Fort Detrick, Maryland 21702-5012

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8. PERFORMING ORGANIZATION REPORT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Military traumatic brain injury (TBI) typically involves blast exposure, and typically occurs in an environment of extreme stress. One of the most common and debilitating consequences of TBI is post-traumatic headache (PTH). Because both TBI and stress could contribute to PTH, we examine them together and separately, attempting to understand the mechanisms by which they generate PTH, and to develop new treatments for the disorder tailored to both injury and stress mechanisms.

In the first year of this project, a significant portion our time has been spent in the ACURO approval process. We obtained ACURO approval in the middle of the third quarter of the calendar year. Prior to ACURO approval (on an unfunded basis) we set up the blast apparatus in our lab and began validation work in this new setting. Since ACURO approval, we have continued validation work for all of the modalities we will need in the project (blast, sensory physiology, and behavior) and begun experiments.

15. SUBJECT TERMS
traumatic brain injury, blast injury, post-traumatic headache, stress

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1. INTRODUCTION:

Post-traumatic headache (**PTH**) is an epidemic in our military personnel. It is a chronic, migraine-like headache that is extremely difficult to treat, whose mechanisms are essentially unknown. For our military personnel PTH also comes at a time of significant stress. Both TBI and stress are risk factors for chronic headache. They may contribute separate or overlapping mechanisms, and treatment can be geared to either or both. ***The goal of this proposal is to determine the respective roles of traumatic brain injury and combat-related stress in mouse models of post-traumatic headache. This work will help understand mechanisms of PTH, and test practical, field-deployable PTH treatment.***

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

traumatic brain injury, blast injury, post-traumatic headache, stress

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Examine the effects of TBI and stress on sensory and affective networks.
Specific Aim 2: Examine the effects of TBI and stress on PTH-relevant behavior.
Specific Aim 3: Use newly developed PTH behavioral models to develop practical treatments.

Milestone(s) Achieved	Timeline	Actual
Local IRB/IACUC Approval	3	3
HRPO/ACURO Approval	6	9.5
Collect experimental data for Major Task 1	18	ongoing

What was accomplished under these goals?

1. Major activities:

Specific Aim 1: Examine the effects of TBI and stress on sensory and affective networks.
Specific Aim 2: Examine the effects of TBI and stress on PTH-relevant behavior.

2. Specific Objectives:

Specific Aim 1.
-Move blast apparatus to Brennan lab, validate it in new setting.
-Establish sensory stimulation and recording protocols.
Specific Aim 2.
-Establish behavioral models that will be used in the proposal.

3. Significant Results/Key Outcomes:

1. Blast Model

Introduction:
The majority of military TBI is blast injury. Along with combat-related stress, blast TBI is specific to military PTH. However phenotypes from blast injury can be elusive. Our collaborators have refined a gun-barrel blast injury model designed to produce replicable diffuse injury consistent with blast TBI in humans. We have moved the blast equipment to our space and have begun to characterize this model in our new location, performed by new personnel.

Methods:
Blast waves are produced by firing large rifle primers (350 Magnum Primers, CCI-Ammunition) seated in an otherwise empty .308 casing modified to accommodate the larger primer size. Animals (n=10) were anesthetized with isoflurane and supported in a Kevlar sling to minimize lung injury. Peak pressures were varied by using a 3-way positioner to place the animal's head relative to the end of the barrel (e.g. 340 kPa at 1.5 cm, along a line 60 degrees from the barrel axis). On-axis blasts were also tested to compare injuries to off-axis blasts. Ten animals were divided between blast treatments (sham: n=2, on-axis at 2.5 cm: n=2, on-axis at 5 cm: n=3, off-axis 60° at 1.5cm: n=3).

Animals were immediately sacrificed and transcardially perfused with cold phosphate buffered saline (PBS) followed by a formaldehyde solution (Formal-Fixx). After the brain was removed, it was stored in Formal-Fixx for 24 hours, then stored in PBS until it was sectioned and stained. Coronal sections (100 μm) were cut by vibratome and every fifth slice was placed in block (4% goat serum, 0.1% Triton-X, 0.5% sodium azide in PBS) for 1 h. Immunoglobulin G (IgG) extravasation was used to detect displaced plasma in brain tissue (Biotinylated GtxRt IgG1 1:500 for 24 h; and Streptavidin-conjugated AlexaFluor-555 1:500, 24 h). Sections were imaged with a confocal laser-scanning microscope (Zeiss; LSM700) using a 10x objective (0.5x digital zoom). Images were analyzed using a thresholding script written for ImageJ to measure lesion count, size, and location within the section.

Results:

Analysis of IgG staining shows evidence of vascular injury in blast-treated animals. As expected, we did not see significant injury in sham-treated animals (Figure 1, Figure 2).
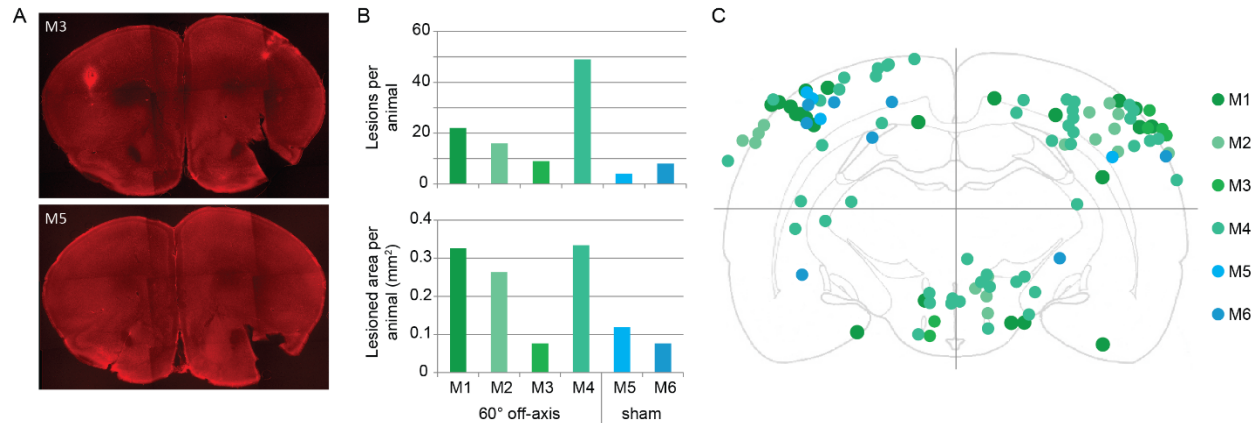

Figure 1: Original blast dataset from collaborators' laboratory. (A) Sample sections from blast-injured (top) and sham-treated (bottom) mice. Lesions were stained for IgG extravasation with Goat anti-Mouse IgG (H+L) secondary antibody biotin-XX conjugate and streptavidin AlexaFluor 555 conjugate. (B) The number of lesions (top) per brain (extrapolated to the entire brain volume). Total lesion size relative to the entire brain (bottom). Blast-injured mice (green tones) show more lesions and have a higher volume of injured brain compared to shams (blue tones). (C) All counted lesions from imaged tissue.
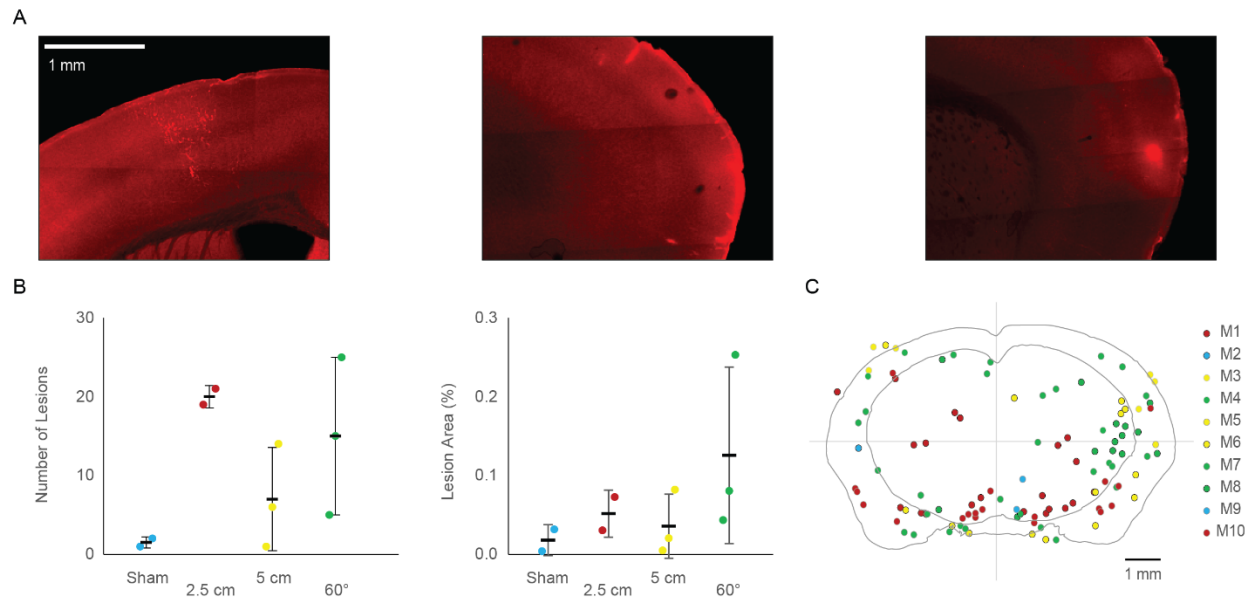

Figure 2: Replication/validation dataset from our laboratory. (A) Sample sections from blast-injured mice showing the types of lesions generated. There are localized stained vessels (left), suggesting vascular damage sufficient enough to affect perfusion upstream from the imaged slice. There are also areas of IgG extravasation that appear near (middle) or below (right) the surface of the cortex. These are areas where IgG is no longer confined to the vessels and suggests a significant breakdown of the blood brain barrier. (B) Plots show the area and number of lesions relative to the imaged sections. Sham animals have few and small lesions, whereas blast-treated animals have more lesions of a range of sizes (black bars are mean+/-std). (C) Scatter plot of lesion location for all animals. Outlines are of a sample brain, 2 mm anterior to bregma (small contour) and 0.5 mm poster to bregma (large contour).

2. Sensory stimulation and recording

Introduction:
It is clear that TBI is associated with sensory network disruptions; PTH is the most obvious example of this. Psychometric testing in humans suggests both defects in sensory processing and increases in sensory gain. Sensory processes appear to undergo biphasic changes after TBI. Spontaneous activity, excitatory synaptic transmission, and evoked sensory response are decreased acutely; however evoked sensory responses are then increased subacutely. *We hypothesize that TBI, stress, or both, cause changes in sensory and affective processing that lead to PTH.*

Cortical spreading depression (CSD) is the physiological correlate of the migraine aura, but it also occurs during TBI – in our work with the controlled cortical impact TBI model, we observed CSD with every episode (CDMRP PR100060). CSD also induces plastic changes in sensory cortical function that might serve as mechanisms entraining PTH. *We use two-photon microscopy and electrophysiology during sensory stimulation and CSD to dissect the cellular and network mechanisms of PTH.*

Methods:
Experiments were performed in awake and head-fixed mice mounted on a floating spherical treadmill. Each mouse was subjected to air puffs to the contralateral whisker pad every 33 seconds for 10 minutes prior to CSD and up to 60 min post-CSD. Neural responses are visualized using the genetically encoded Ca^{2+} indicator GCaMP6f, delivered to the barrel cortex by injecting adeno-associated viruses. Visualization of neural responses is performed using an implanted cranial window and 2-photon microscopy. CSD is induced by topical application of 1M KCl through a second burr hole in the skull.

Results:
Following CSD, neural Ca^{2+} responses within layer 2/3 of the barrel cortex show increased amplitude during whisker stimulation. Further, an increased proportion of neural responses occur during whisker stimulation, rather than during a latency period following stimulation. This provides preliminary evidence that CSD induces complex changes in neural network processing and provides potential network level mechanisms that underlie the changes in sensory perception seen in PTH.
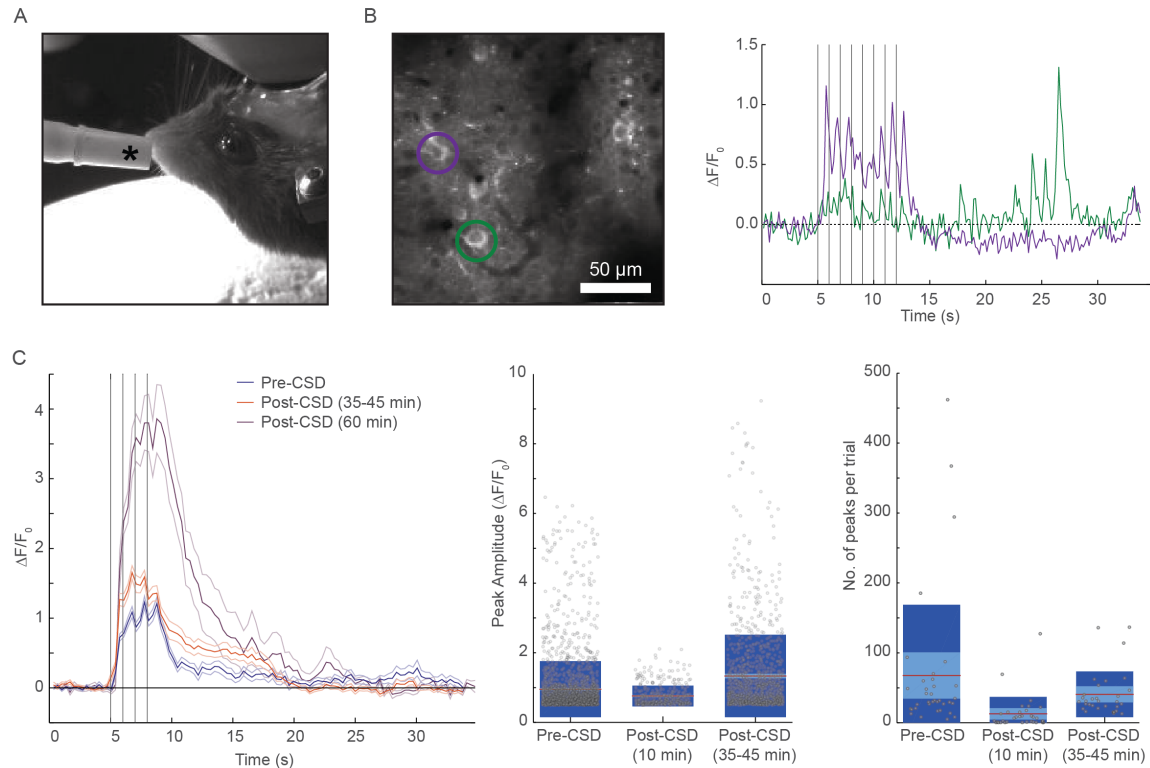
Figure 3: (A) Whisker stimulation in awake behaving mouse. Air source denoted by asterisk. (B) GCaMP6f image during awake recording from layer 2/3 neurons. Trace shows Ca2+ responses from cells circled in image. Vertical bars indicate 1Hz whisker stimulation. (C) Left: Traces of neurons that respond preferentially during whisker stimulation averaged from one animal pre-CSD (10 min period), 30 minutes post-CSD (9 min period), and 60 minutes post-CSD (3 min period). Middle: Comparison of peak amplitude; One-way ANOVA, p = 2.1436-42; p <= 0.0001 for all comparisons. Right: Frequency of peak events per trial; One-way ANOVA, p = 0.0027; p = 0.0018 for Pre-CSD vs Post-CSD (10 min). Bonferroni correction used for all comparisons. Repeated measures ANOVA, n.s. for all figures. N = 2 animals; Pre-CSD: 36 trials (including both animals), 2,434 peaks; Post-CSD (10 min): 34 trials, 435 peaks; Post-CSD (35-45 min): 31 trials, 1,262 peaks.

We have developed an experimental paradigm to study the effects of TBI-associated CSD on sensory processing in awake mice. Neural responses to whisker stimulation are more consistent in awake mice, underlining the importance of studying the effects of CSD in an awake state. In the wake of CSD, neural Ca^{2+} responses are less frequent and have decreased amplitude. Thirty-five minutes after CSD, the frequency of responses during whisker stimulation returns and the average amplitude of Ca^{2+} responses is increased compared to before CSD.

3. Behavioral models of stress, sensory and affective response

Introduction:
Precise circuit alterations are translationally meaningless without relevant correlated behavior. PTH manifests in humans as pain behavior that completely disrupts function. It is also comorbid with stress and affective disorders. A major portion of our research effort has been dedicated to measuring behavioral endpoints in mice, so that they can be meaningfully used to study PTH. Given the coincidence of TBI and stress in the origins of military PTH, we are interested in dissecting the influence of each.

Chronic variable stress model: All animals had their weight measured before and on the last day of chronic stress paradigm to show that chronically stressed mice had a significant reduction in weight gain after but not before the stress paradigm (Figure 4). Females were also evaluated daily for their phase in the estrous cycle using vaginal lavage. Stressed females showed a significant reduction in their number of cycle during the 40 days variable stress paradigm compared to their control counterparts (Figure 5).
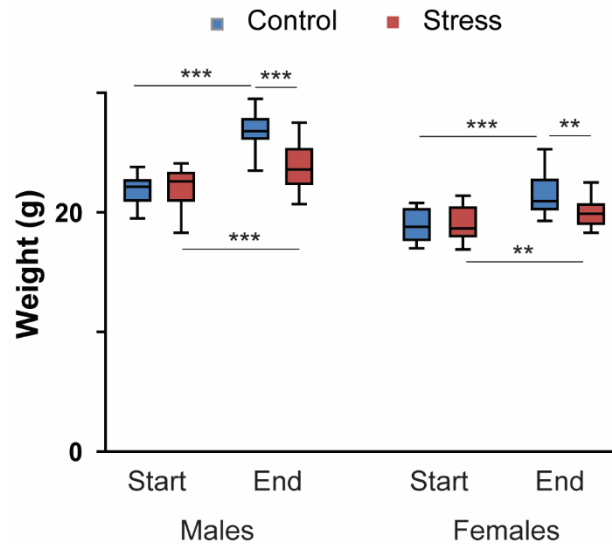

Figure 4: Box whisker plots representing animal weights measured before (start) and after (end) the 40 day chronic variable stress paradigm for both males (left panel) and female (right panel) mice. No difference was seen between stress and control groups before start of the stress paradigm. However stress mice, both male and female, showed a significant reduction in expected age-associated weight gain at the end of the stress paradigm compared to their control counterparts. (** p<0.01, *** p<0.001, Tukey multiple comparison test, n-22, 21 for males control and stress respectively; n=16 per group for females).
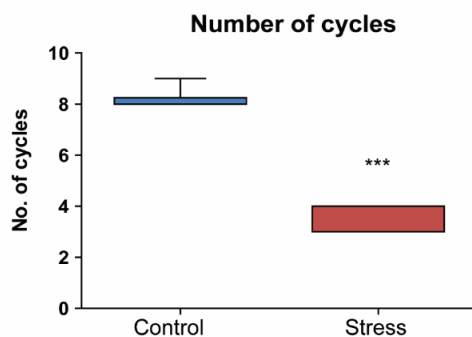

Figure 5: Box whisker plots representing the number of cycles during 40 days chronic stress. A cycle was defined as a transition between the diastrous phase to the proestrous phase. Stressed females showed a significant decrease in number of cycles. (*** p<0.001, Student's t-test, n=6 per group for females).

At the end of the chronic variable stress paradigm, the animals were evaluated for locomotion/exploration and anxiety measures using the open field test and elevated plus maze

test. In the open field test all animals were allowed to explore a large circular arena (110 cm diameter x 38 cm height) for 30 min. Both chronically stressed male and female mice showed an increase in anxiety measures as measured by their reduced time spent in the center of the arena (Figure 6). However only chronically stressed males showed increased locomotion as measured by an increase in: total distance travelled, length of progression segments and maximal speed (Figure 6). Chronically stressed female mice did not show a difference in locomotion parameters.
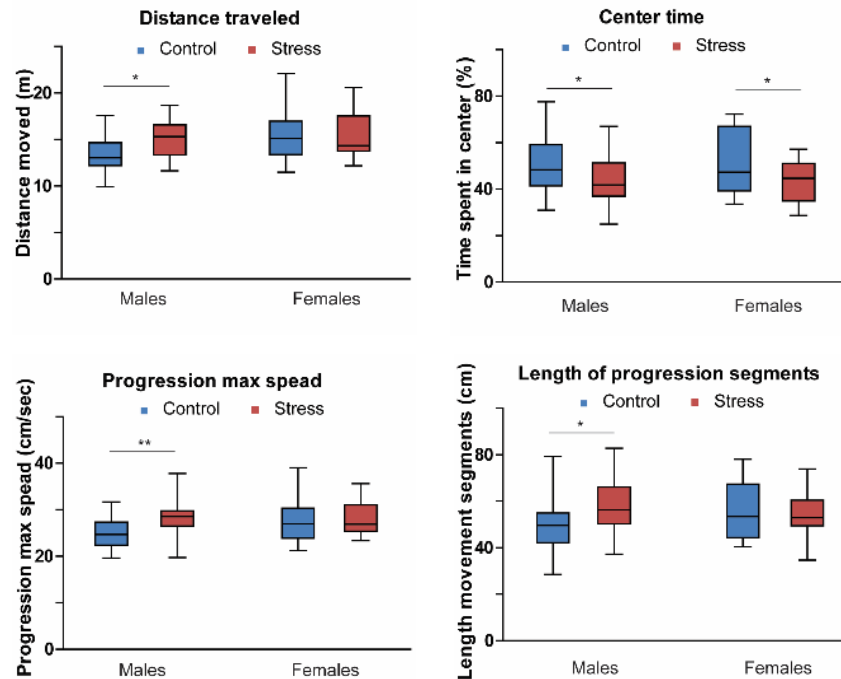

Figure 6: Box whisker plots representing the activity of both chronically stressed males and females vs. their control counterparts in the open field test. Upper left: distance moved during 30 min test. Chronically stressed males but not females, showed an increase in distance moved vs. their control counterparts. Upper right: Time spent in the center of the open field arena. Both chronically stressed males and females showed a reduction in time spent in the center of the arena indicating an increased stress response. Lower left: Maximal speed in progression segments. Chronically stressed males but not females, showed a significant increase in maximal speed travelled compared to controls. Lower right: length of progression segments. Chronically stressed males but not females showed an increase in the length of progression segments performed in the open field test. These results indicate increased anxiety measures in both chronically stressed males and females compared to controls. Chronically stressed males but not females showed an increase in locomotor measures as well. (*p<0.05, ** p<0.01, Student's t-test, n=22, 21 in males and n=16 per group in females for stressed vs controls respectively.

We further strengthened our results with the elevated plus maze (EPM), a separate anxiety measure. In the EPM, animals were allowed to explore the open arms (5cm x 5 cm x 35 cm) and the closed arms (16cm x 5 cm x 35 cm) for 30 min. Both chronically stressed males and females showed an increase in time spent in the closed arms and a decrease in time spent in the open arms (Figure 7).

We then measured the effect of chronic stress on sensory function, measuring mechanical allodynia before and after administration of nitroglycerine (NTG), using von Frey filaments (VFF) in a modified "up-and-down method" paradigm. NTG infusion triggers migraine without aura in human migraineurs and reduced mechanical allodynia threshold in rodents. This enables

NTG to be used to generate headache-relevant pain endpoints in rodent models. Chronically stressed animals showed a significant reduction in baseline mechanical allodynia threshold compared to their control counterparts (Figure 8). After administration of NTG, both chronically stressed males and females showed a significant reduction in mechanical allodynia compared to baseline, similarly to their control counterparts (Figure 8). We also evaluated the effects of chronic stress on CSD susceptibility. Chronically stressed males did not show a significant difference in CSD frequency compared to their control counterparts, however chronically stressed females showed a significant increase in CSD frequency, tied to their estrous phase (Figure 9).
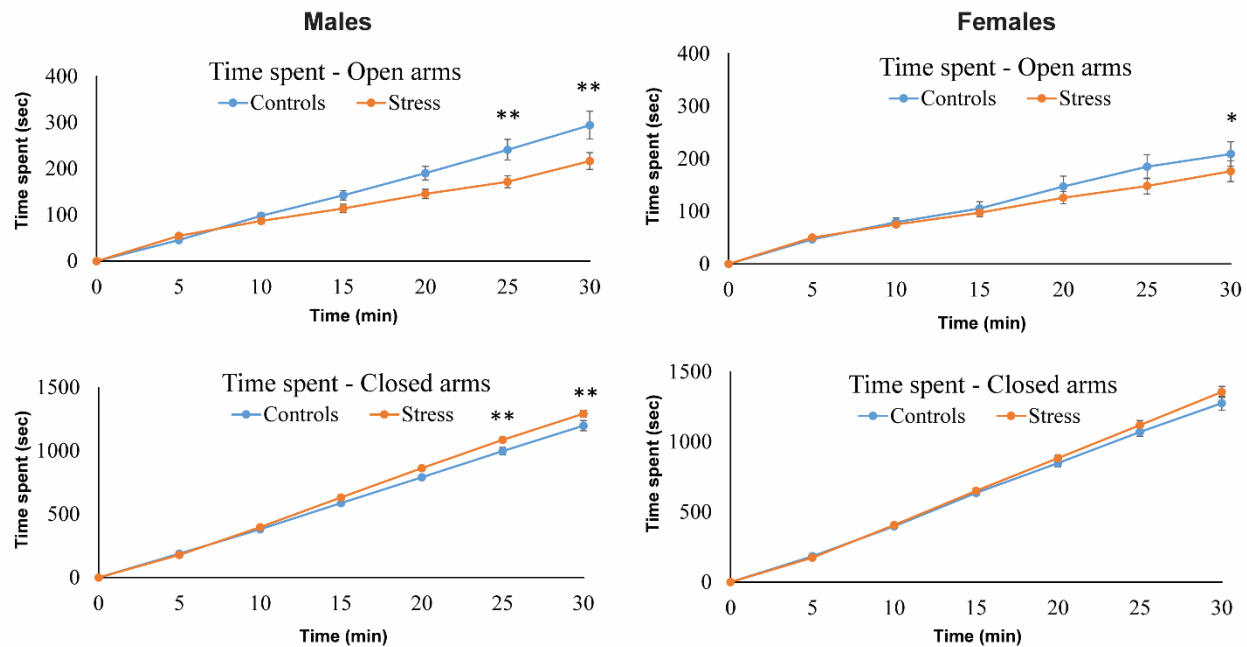

Figure 7: Graphs indicate cumulative time spent in open arms (upper graphs) and closed arms (lower graphs) in males (left graphs) and females (right graphs) chronically stressed (orange) vs. controls (blue). Both chronically stressed males and females showed a significant reduction in time spent in open arms and an increased in time spent in the closed arms as seen after 25 and 30 min for males and 30 min only for females. (*p<0.05, ** p<0.01, Kolmogorov-Smirnov Test followed by Bonferroni multiple comparison test, n=22, 21 for males and n=16 per group for females control vs. chronic stress respectively).
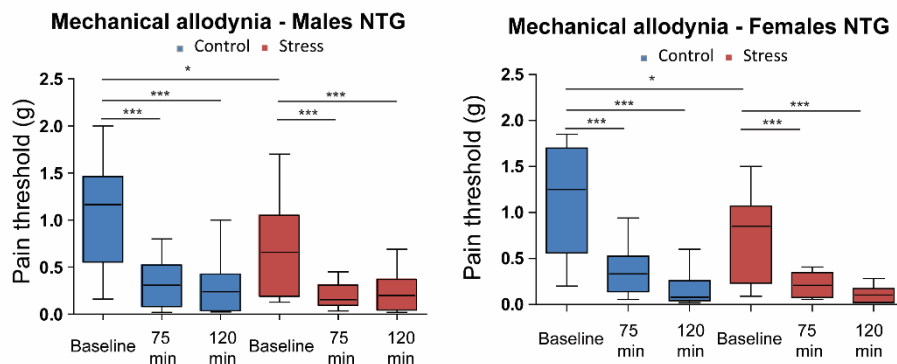

Figure 8: Box whisker plots represent mechanical allodynia evaluataed before (baseline) and 75 and 120 min after administration of NTG for males (left graph) and females (right graph) chronically stressed (red box) and controls (blue box).

Baseline threshold was significantly reduced in chronically stressed males and female mice compared to their control counterparts. Both stressed and control males and females showed a similar and significant reduction in baseline threshold 75 and 120 min after administration of NTG. (*p<0.05, *** p<0.001, Tukey multiple comparison test, n=16, 15 for males and n=13 per group for females control vs. chronic stress respectively).
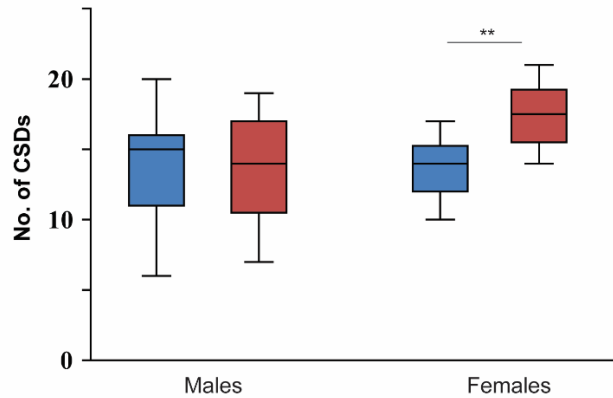

Figure 9: Box whisker plots represent the number of CSDs evaluated for chronically stressed (red) vs. control (blue) for male and female mice. Chronically stressed males did not show any difference in no. of CSDs compared to their control counterparts. Chronically stressed females showed a significant increase in number of CSDs compared to controls. This indicates an increased hyperexcitability for stressed females but not males thereby indicating a migraine relevant phenotype. (** p<0.01, Student's t-test, n=22, 21 for males and n=10 per group for females control vs. chronic stress respectively).

These results indicate that our chronic variable stress paradigm was able to produce a measurable stress response compared to control animals. Both males and females showed a reduction in pain threshold after the stress paradigm, but only females showed an increased cortical excitability (evidenced by increased CSD susceptibility).

4. Other Outcomes

Nothing to report.

What opportunities for training and professional development has the project provided?

Undergraduate training:
-Jarem Kilby
-Nicholas McKean
MD Training:
-Natalie Rea, MD candidate
-Austin Service, MD candidate
PhD/Graduate Training:
-Pratyush Suryavanshi, PhD candidate
Postdoctoral Training:
-Punam Sawant, PhD
-Dan Kaufmann, PhD
-Jeremy Theriot, PhD

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Specific Aim 1: Examine the effects of TBI and stress on sensory and affective networks.	Timeline	Actual
Major Task 1: Allocate to stress or control groups, perform TBI or sham, perform two-photon microscopy and *in vivo* whole cell recordings.	6-24	10-30
Specific Aim 2: Examine the effects of TBI and stress on PTH-relevant behavior.		
Major Task 1: Allocate to stress or control groups, perform TBI or sham, perform nitroglycerin and photophobia testing	6-24	10-30

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Optimization of blast injury protocols for mice – this is a relatively undeveloped field so standardization is important.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report – however it is possible that our standardized blast models could be commercializable in the future.

What was the impact on society beyond science and technology?

Nothing to report.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

1. ACURO approval was delayed nearly four months relative to anticipated approval; this has delayed our experimental timeline proportionately.

2. We moved the blast apparatus from Co-Investigator Dr. Monson's lab to our lab. This will significantly increase productivity in the long term. However in the short term we have had to do more validation work to make sure the device is performing as expected in its new setting.

Changes that had a significant impact on expenditures

We have had to delay hiring staff due to delayed ACURO approval, however we are now fully staffed.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

Kaufmann D, Bates EA, Yagen B, Bialer M, Saunders GH, Wilcox K, White HS, **Brennan KC** (2015). *sec*-Butylpropylacetamide (SPD) Has Anti-Migraine Properties. *Cephalalgia, In press. Federal support acknowledged.*

Books or other non-periodical, one-time publications.

Ayata C, **Brennan KC**. Animal models of migraine aura. In *The Neurological Basis of Migraine.* NY Academy of Sciences/Wiley. *Pending. Federal support acknowledged.*

Other publications, conference papers, and presentations

Nothing to report.

Website(s) or other Internet site(s)

http://medicine.utah.edu/neurology/research/headache-physiology-lab/

Technologies or techniques

Nothing to report – however it is possible that our standardized blast models could be commercializable in the future.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *KC Brennan*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: *1*
Contribution to Project: *Dr. Brennan supervised the project.*
Other support: *NIH NS085413, clinical and departmental income.*

Name: *Jeremy Theriot*
Project Role: *Postdoc*
Researcher Identifier (e.g. ORCID ID): N/A

Nearest person month worked: 1
Contribution to Project: Dr. Theriot worked on moving, testing and validating blast apparatus in its new location.
Other support: NIH NS085413.

Name: Natalie Rea
Project Role: Medical Student Researcher
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1
Contribution to Project: Ms. Rea worked on behavioral experiments.
Other support: NIH NS085413.

Name: Dan Kaufmann
Project Role: Postdoc
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1
Contribution to Project: Dr. Kaufmann worked on behavioral experiments.
Other support: NIH NS085413.

Name: Pratyush Suryavanshi
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1
Contribution to Project: Mr. Suryavanshi began electrophysiology experiments.
Other support: NIH NS085413.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

> Nothing to report.

What other organizations were involved as partners?

> Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**

   QUAD CHART: Submitted with attachments.

9. **APPENDICES:** Nothing to report.